Plant promoter and utilization thereof

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Abstract of **EP0824150**

There are disclosed a plant promoter which is functional in plant cells comprising a nucleotide sequence (abut 250 bp) of SEQ ID NO: 1 and use thereof.

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(54) **Plant promoter and utilization thereof**

(57) There are disclosed a plant promoter which is functional in plant cells comprising a nucleotide sequence (abut 250 bp) of SEQ ID NO: 1 and use thereof.

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Description

The present invention relates to a plant promoter and utilization thereof.

The cauliflower mosaic virus 35S promoter (hereinafter, referred to as 35S promoter) has been known as an effective promoter for non-tissue-specific expression of a desired protein gene in plant cells and has been widely used.

However, an effective promoter that enables tissue-specific expression, particularly in root, of a desired protein gene in order to produce desirably transformed plants has been desired.

Accordingly, the technical problem underlying the present invention is to provide a promoter capable of functioning in plant cells, which enables tissue-specific expression of a gene of interest in vascular bundles, particularly vascular bundles in the root of plants.

This technical problem is solved by the embodiments characterized in the claims. Thus, the present invention relates to

1. A promoter which is functional in plant cells, comprising a nucleotide sequence (about 250 bp) of SEQ ID NO: 1,
2. A plasmid comprising the promoter of SEQ ID NO: 1.
3. A gene coding for a protein having a molecular weight of 16 kD, having a nucleotide sequence encoding an amino acid sequence of SEQ ID NO: 3,
4. A gene coding for a protein having a molecular weight of 16 kD, having a nucleotide sequence of SEQ ID NO: 4, and
5. A terminator capable of functioning in plant cells comprising a nucleotide sequence of SEQ ID NO: 5.

Fig. 1 shows pCR16G1/250-GUS, pCR16G1/EV-GUS and pCR16G1/H-GUS which are the plasmids of the present invention.

Fig. 2 shows pCR16G1/Xb which is a plasmid containing the gene of the protein of the present invention.

Fig. 3 shows a comparison between the amino acid sequence of the protein of the present invention and amino acid sequences of various proteins.

Fig. 4 shows pCR16G1/Xh which is a plasmid of the present invention (a plasmid containing a part of the gene of the protein of the present invention and the promoter of the present invention).

Fig. 5 shows steps for constructing pCR16G1/250-GUS, which is a plasmid of the present invention, from pBI101.

Fig. 6 shows steps for constructing pCR16G1/EV-GUS, which is a plasmid of the present invention, from pBI101 and pCR16G1/250-GUS.

Fig. 7 shows steps for constructing pCR16G1/H-GUS, which is a plasmid of the present invention, from pBI101.

Fig. 8 shows a promoter of the present invention, which has a region (about 4 Kbp) having restriction sites for XhoI (0 kb), XbaI (0.3 kb), EcoRV (2 kb), EcoRV (2.3 kb), EcoRI (3 kb), SmaI (3.6 kb) and HindIII (4 kb).

Fig. 9 shows a visualization (showing morphology of an organism) by staining indicating high expression of the desired protein in the vascular bundles of a plant owing to the promoter of the present invention. The vascular bundles are white in this drawing as the result of staining. In the drawing, the black area is the non-stained region and the white area is the stained region.

Fig. 10 shows a visualization (showing morphology of an organism) by staining indicating high expression of the desired protein in the vascular bundles of a plant owing to the promoter of the present invention.

In the drawing, the black area is the non-stained region and the white area is the stained region, the vascular bundles are white in this drawing as the result of staining.

The present invention viii be described in more detail.

In the present invention conventional gene engineering methods described, for example, in J. Sambrook, E. F. Frisch and T. Maniatis, Molecular Cloning, 2nd. Ed., published by Cold spring Harbor Laboratory Press, 1989; D. M. Glober, DNA Cloning, published by IRL, 1985; and so on can be used.

First, description will be made to the promoter which is functional in plant cells comprising a nucleotide sequence as depicted in SEQ ID NO: 1.

The promoter may comprise a nucleotide sequence as depicted in SEQ ID NO: 2, which includes the nucleotide sequence as depicted in SEQ ID NO: 1.

The promoter may also comprise a nucleotide sequence (about 4 Kbp) having the following characteristics:

a. isolated and/or purified from carrot;
b. having restriction enzyme sites for XhoI (0 kb), XbaI (0,3 kb), EcoRV (2 kb), EcoRV (2.3 kb), EcoRI (3 kb), SmaI (3.6 kb) and HindIII (4 kb); and
c. containing a nucleotide sequence as depicted in SEQ ID NO: 2.

The promoters are preferably used for root-specific expression of a desired structural gene.

The "promoter which is functional in plant cells" herein mean a promoter having an ability of controlling expression of a protein in plant cells when a structural gene of the desired protein is ligated downstream of said promoter.

The promoters of the present invention as described above may be further modified by ligating to a nucleotide sequence such as:

a transcription-translation activating sequence formed by ligating the -333 to -116 region of the Agrobacterium octopine synthesis gene with the -318 to -138 region of the mannopine synthase gene,
a transcription-translation activating sequence formed by ligating the -318 to -213 region of the mannopine synthase gene with -333 to -116 region of the octopine synthesis gene (The Plant Journal, 7(4), 661 - 676 (1995)),
a nucleotide sequence containing the -343 to -91 region of the cauliflower mosaic virus 35S promoter (Nature, 313, 810 - 812 (1985)),
a nucleotide sequence containing the -1099 to -205 region of tomato ribulose-1,5-diphosphate carboxylase oxylase small subunit gene (rbc-3A) (Plant Cell, 1, 217 - 227 (1990)),
a nucleotide sequence containing the -902 to -287 region of tobacco PRla gene (Plant Cell, 2, 357 - 366 (1990)), or
a nucleotide sequence containing the -1300 to -195 region of potato protease inhibitor gene (PI-II) (Plant Cell, 2, 61 - 70(1990)).

The plasmid having anyone of the promoters of the present invention is usually constructed to have one or more cloning sites for inserting or excising a desired structural gene downstream of the promoter of the present invention.

The cloning site herein means a site which can be recognized and cleaved by a restriction enzyme used in the genetic engineering technology.

More specifically, the plasmid also comprises a chimeric gene which is prepared by ligating a structural gene of a desired protein downstream of the promoter (the structural gene may be heterologous in relation with the promoter) in order to express a desired protein in plant cells.

Such chimeric gene includes, for example,

(1) a recombinant DNA gene having only the promoter of the present invention and the desired structural gene, and
(2) a plasmid such as an extrachromosomal gene which contains said recombinant DNA gene and which is autonomously replicating physically independent of chromosomes of host and stably inheritable, and the like.

For enhancing the expression efficiency of the desired protein, plant cells are preferably transformed with a plasmid having the promoter of the present invention, the desired structural gene and a terminator which is functional in plant cells.

The "terminator which is functional in plant cells" means that the terminator has an ability of effectively terminating transcription of the desired structural gene in plant cells. Such a terminator usually exist in a genomic DNA region located downstream of a poly A sequence which usually exists downstream of poly (A) added-signal (made into a consensus sequence with AATAAA) present in a 3'-terminal non-translational region located downstream of a termination codon in structural genes of various proteins.

Examples of the terminator include a terminator which is functional in plant cells containing a nucleotide sequence as depicted in SEQ ID NO: 6, a terminator (NOS) in nopaline synthase gene derived from a plant gene, a terminator of garlic virus GV1, GV2 genes or the like.

Specific examples of the plasmid of the present invention having a cloning site include the plasmid shown in Fig. 1: pCR16G1/250-GUS, pCR16G1/EV-GUS and pCR16G1/H-GUS or the like.

The plasmid of the present invention can be prepared, for example, by the following process.

First, the promoter comprising a nucleotide sequence as depicted in SEQ ID NO: 1 is inserted in a multicloning site of a plasmid containing a terminator which is functional in plant cells, for example, pBI101 (manufactured by Clontech) (Jefferson et al., EMBO J., 6, 3901 - 3907 (1987)).

Then, an exogenote such as an existing marker gene, for example β-glucuronidase gene (hereinafter, referred to as GUS gene) is excised and said exogenote is replaced by a desired structural gene.

Alternatively, the promoter of the present invention, a desired structural gene and a terminator which are functional in plant cells are inserted in this order into a multicloning site of a binary vector, for example, pBIN19 (Nuc. Acid Res., 12, 8711 - 8721 (1984)).

Preferred examples of desired structural genes contained in the chimeric gene of the present invention include useful genes capable of enhancing resistance against damage by disease and pest.

Examples of such a useful gene include plant protection genes such as phenylalanine ammonia-lyase gene (PAL), chalcone synthase gene (CHS), chitinase gene (CHT), lysozyme gene, PR protein gene and the like, and disease resistance genes such as Pto gene, viral coat protein gene and the like.

Combat against pathogenic fungi localized in plant vascular bundle and pests taking nutrient from vascular bundle

region or the like can be efficiently made by good expression of proteins encoded by these useful genes in vascular bundle of a plant utilizing the promoter of the present invention.

Further, since the promoter of the present invention enables expression of a desired protein particularly in vascular bundles of root, it is useful in

(1) combat against pathogenic soil fungi and pests which are difficult to kill by chemicals and creation of a functional crop plant of which disease-susceptible root is provided with immunity or resistance against pathogenic fungi and pests; and
(2) improving nutritive value of edible root plants, or creating of crop plant having an increased content of nutrients such as proteins.

Desired structural genes used for such purpose include, for example, BT (*Bacillus thuringiensis*) toxin protein gene,

genes capable of enhancing resistance against damage by disease and pest as described above,
genes capable of increasing content of various proteins in feeding crops such as storage protein gene including conglycinin gene, β-conglycinin gene of soybean and the like,
genes capable of increasing methionine content or lysine content in feeding crops such as 2S albumin gene of Bertholletia excelsa Humb., 10 kDa and 15 kDa protein genes of corn and rice and the like,
genes associated with biotin biosynthesis and are capable of increasing biotin content in feeding crop plant such us bioA, bioB, bioC, bioD, bioF or bioH enzyme genes of microorganism including *Escherichia coli* and the like,
genes capable of increasing oxidation stability of lipids and of improving lipids by decrease of phospholipid and increase of oleic acid and linolenic acid such as genes for stearoyl-ACP-desaturase, acyl-ACP-thioesterase, 3-phosphate acyl transferase and the like,
genes capable of increasing resistance against low temperature by increase of a ratio of unsaturated fatty acids such as acyl transferase gene,
as well as genes which are herbicide resistance-associated genes and are capable of creating herbicide resistant crops such as genes of L-phosphonothrisine acetylase, (EPSP) synthase, PPO and the like.

Methods for introducing the chimera gene or the plasmid of the present invention include, for example, known methods such as *Agrobacterium* method (a method in which *Agrobacterium*, a soil bacterium, is infected to a plant tissue), electric introduction method (a method of electric introduction into protoplast: electroporation), direct introduction method by particle gun (a direct introduction method into plant tissue or cultivated cells: particle gun method) and the like. The plant cells transformed with the promoter, chimeric gene or plasmid of the present invention can be regenerated by conventional plant tissue cultivating techniques described, for example, in S. B. Gelvin, R. S. Schilperoot and D. P. S. Verma, Plant Molecular Biology, Manual, Kluwer Academic Publishers Press (1998); Valvekens et al., Proc. Natl. Acad. Sci., 85, 5536 - 5540 (1988) to give a plant or a part thereof originated from said plant cells.

Plants usable in the present invention include, for example, monocotyledonous such as rice, maize, barley, wheat, onion and the like,

dicotyledonous including *Leguminosae* plant such as soybean, pea, bean, alfalfa and the like,
Solanaceae plants such as tobacco, tomato, potato and the like,
Cruciferae plants such as cabbage, rape, mustard and the like,
Cucurbitaceae plants such as melon, pumpkin, cucumber and the like,
Umbelliferae plants such as carrot, celery and the like, and *Compositae* plants such as lettuce and the like.

Plant cells and plants into which a desired structural gene has been introduced and expressing a protein encoded by the desired structural gene under control of the promoter of the present invention can be obtained in the above described manner.

The present invention further provides a protein which is isolated and /or purified from the root of carrot (*Daucus carota L.*) such as Kuroda Gosun. The protein has a molecular weight of 16 kD, of which amino acid sequence is shown in SEQ ID NO: 3. The nucleotide sequence depicted in SEQ ID NO: 4 represents a nucleotide sequence of genomic DNA which contains both intron region and cDNA sequence coding for the amino acid sequence as depicted in SEQ ID NO: 3.

According to the present invention, creation of a carrot which is rich in a certain protein nutrient of interest becomes possible by introducing and expressing a gene encoding the protein into the root, for example, by the method described above utilizing the promoter of the present invention.

Similarly, the nutritive value of root crops can be improved by introduction and expression of a gene encoding a nutrient protein in the root. Examples of root crops include, for example, radish, turnip, sugar beet, burdock and the like.

Further, when a useful protein or its nucleotide sequence having a high homology with the protein or the nucleotide sequence encoding the protein of the present invention can be found in nucleotides data base such as EMBL, NBRF and so on, the nucleotide sequence that codes for the useful protein can be readily obtained by a conventional hybridization and cloning technique using the gene of the present protein, therefore the thus obtained gene can be introduced and expressed effectively by the present promoters.

Examples of such useful proteins include:

(1) a protein contributing to resistance against pathogens, which can provide a plant with resistance against pathogens and is useful for clarifying a mechanism of resistance against pathogen;
(2) a protein which acts as a plant hormone, and is expressed or induced in varying amount under influence of stress from the outside world or the like, of which expression control enables various improvements in cultivar utilizing response to stress or hormone;
(3) a protein associated with pollen allergy; controlled expression of the protein enables improvements in cultivar such that it forms improved pollen or controlled expression amount of pollen, which may result in no or low allergy; and
(4) a protein associated with heat shock, which enables improvement in retaining or transport of plants by assisting holding of useful proteins.

Methods for isolating the promoter, terminator and protein of the present invention are illustrated below.

First, a genomic DNA is prepared from leaves, partially hydrolyzed with an appropriate enzyme and ligated to a vector arm derived from a λ-phage. This is packaged *in vitro* to form a phage particle, which is infected to *Escherichia coli* resulting in a plaque on an agar medium. This is recovered and a genomic library therefrom is used for gene screening. Methods for preparing genomic DNA include, for example, CTAB method described in M. Shure et al., Cell, 35, 325 (1983), the urea-phenol method described in S. O. Rogers and A. J. Bendich, Plant. Mol. Biol., 5, 69 (1985) and the like. As the λ-vector, for example, λ FIX II, λ EMBL3, λ EMBL4, λ DASH II available from Stratagene and the like can be used.

For *in vitro* packaging, for example, Gigapack Packaging Extract available from Stratagene can be used.

For selecting a genomic clone containing the nucleotide sequence of the promoter, the terminator or the protein of the present invention from the genomic library, an effective method is, for example, plaque hybridization using a probe formed by labelling a cDNA corresponding to a desired gene or a cDNA similar to the desired gene with RI or a fluorescent reagent or the like. The RI labelling can be effected, for example, using Random Labelling Kit available from Boeringer or Takara Shuzo or the like.

The fluorescent labelling can be effected, for example, using ECL Direct Nucleic Acid Labelling and Detection System available from Amersham or the like.

The genomic clone containing the nucleotide sequence of the promoter of the present invention, the terminator of the present invention or the protein of the present invention obtained by screening can be sequenced by subcloning into a plasmid vector of which DNA preparation or analysis can be conducted by a conventional method, in which commercially available pUC18, pUC19, pBluescript KS+, pBluescript KS- or the like are used to form a plasmid DNA, and a cycle sequence method in which Sanger method described in Sanger et al.,J. Mol. Biol., 94, 441 (1975) and Sanger et al., Proc. Natl. Acad. Sci., 74, 5463 (1977) add PCR described in Saiki et al., Science, 230, 1350 (1985) can be used.

The present invention will now be described in more detail by means of Examples, which should not be construed as a limitation upon the scope of the present invention.

Example 1: Isolation of a gene of the protein of the present invention

A genomic library was made using a genomic DNA prepared from carrot leaves. The genomic library was screened using an already obtained cDNA fragment as a probe and two positive clones were obtained. The screening process is illustrated below:

Step 1: Preparation of a carrot genomic library

(1) Preparation of a carrot genomic DNA

In liquid nitrogen 10 g of carrot leaves were triturated at 6 weeks after seeding. The triturate was suspended in 5 ml of 2 × CTAB solution (2% cetyl trimethyl ammonium bromide, 100 mM Tris-HCl buffer, pH 8.0, 20 mM EDTA, pH 8.0, 1.4 M NaCl, 1% polyvinylpyrrolidone) and incubated at 55°C for 10 minutes. The same amount of chloroform / isoamyl alcohol (24 : 1) was added thereto and they were gently mixed at room temperature for 30 minutes, followed by centrifugation to separate the upper and lower layers.

(a) To the upper layer the same amount of chloroform/isoamyl alcohol (24 : 1) was added, and

(b) to the lower layer the same amount of 1 x CTAB solution was added (a double dilution of 2 × CTAB solution with sterilized distilled water), and they were gently mixed at room temperature for 10 minutes, again followed by centrifugation, upon which the upper layers from both (a) and (b) were taken and mixed. To the mixture 1/10 amount of 10% CTAB solution were added (10% cetyl trimethyl ammonium bromide, 0.7 M NaCl) and the same amount of a precipitation buffer (2% cetyl trimethyl ammonium bromide, 50 mM Tris-HCl buffer, pH 8.0, 10 mM EDTA, pH 8.0) and they were gently mixed, followed by centrifugation. The obtained precipitates were suspended in 1 M NaCl-TE (1 M NaCl, 10 mM Tris-HCl buffer, pH 8.0, 1 mM EDTA, pH 8.0). The same amount of isopropanol was added thereto and they were gently mixed, followed by centrifugation. The obtained precipitates were rinsed with 70% ethanol, dried for a short time and suspended in TE. RNase was added thereto to a final concentration of 10 μg/ml and reacted at 37°C for 30 minutes, upon which 1/4 amount of 4 M ammonium acetate and 2-fold amount of 100% ethanol were added and mixed to precipitate DNA. The obtained DNA was rinsed with 70% ethanol, dried for a short time and suspended in TE (10 mM Tris-HCl buffer, pH 8.0, 1 mM EDTA). The DNA solution was appropriately diluted, assayed for absorbance and electrophoresed in agarose gel to confirm production of about 350 μg of genomic DNA.

(2) Partial hydrolysis and insertion into λ-vector of the genomic DNA

A mixture of a 50 μg portion of the genomic DNA obtained as above and Sau3AI at a final concentration of 0.08 U/μl was incubated at 37°C for 50 minutes to partially hydrolyze the DNA. A portion of the solution was fractionated by 0.5% agarose gel electrophoresis and it was confirmed that it was major in 20 - 50 kb DNA fragments. To this DNA solution an equal amount of phenol/chloroform/isoamyl alcohol (25:24:1) was added and they were sufficiently mixed, followed by centrifugation and separation of the upper layer. The treatment with phenol/chloroform/isoamyl alcohol (25 : 24 : 1) was once repeated and the obtained solution was combined with 1/10 amount of 3 M sodium acetate and twice the amount of 100% ethanol, mixed well and the mixture was centrifuged after cooling at -80°C for 10 minutes. Precipitates were rinsed with 70% ethanol, dried for a short time and suspended in TE. To this DNA solution 167 μM dATP, 167 μM dGTP and Klenow (Takara Shuzo) were added at a final concentration of 0.05 U/μl and the mixture was reacted at room temperature for 15 minutes. To this 1/10 amount of 10 x STE and an equal amount of 1 x STE was added and mixed. To the solution an equal amount of phenol / chloroform / isoamyl alcohol (25:24:1) was added and they were sufficiently mixed, followed by centrifugation and separation of the upper layer. The treatment with phenol / chloroform / isoamyl alcohol (25 : 24 : 1) was once repeated and the obtained solution was combined with 1/10 amount of 3M sodium acetate and twice the amount of 100% ethanol, mixed well and the mixture was centrifuged after cooling at -80°C for 10 minutes. Precipitates were rinsed with 70% ethanol, dried for a short time and suspended in TE. A reaction (50 mM Tris-HCl buffer, pH 7.5, 70 mM $MgCl_2$, 10 mM DTT) containing of a portion of this solution and 1 μg of λ/FIXII vector (Stratagene) was prepared. To this T_4 DNA ligase (Takara Shuzo) was added and the mixture was reacted overnight at 16°C.

(3) Packaging and Amplification of the library

The reaction solution from (2) was packaged using Gigapack II Gold Packaging Extract (Stratagene) and a genomic library having a bank size of 6×10^4. The host, Escherichia coli XL1-Blue MRA (P2), cultured overnight with shaking at 37°C was suspended in 10 mM magnesium sulfate at a cell concentration: $OD_{600} = 0.5$. To 200 μl of the cell suspension was added 10,000 pfu of phage and the mixture was scattered together with top agar preheated to 50°C on a NZY plate, incubated at 37°C for 8 hours and the propagated phage was suspended in SM buffer (50 mM Tris-HCl buffer, pH 7.5, 0.1 NaCl, 7 mM $MgSO_4$, 0.01% gelatine). The phage suspension was harvested and used for the following screening.

Step 2: Screening of the carrot genomic library

(1) Preparation of a filter for screening

On a NZY plate was scattered 50,000 pfu of phage and incubated at 37°C for 8 hours. Onto this plate a nylon filter Hybond-N (Amersham) was overlapped and stood for 1 minute to adsorb the phage. Then the filter was treated with alkali to lyse the phage while DNA was bound to the filter, followed by treatment for neutralization (1.5 N NaCl, 0.5 M Tris-HCl, pH 8.0, 3 minutes × 2 times). This was air-dried after washing with 2 × SSC (300 mM NaCl, 30 mM citric acid)for 5 minutes, and irradiated with UV for 2 minutes to fix the DNA on the filter. The following hybridization reaction was carried out using 12 filters prepared in such manner.

(2) Plaque hybridization

The filters prepared above were placed in Hybripack, combined with a hybridization solution (6 × SSC / 1% SDS / 100 μg/ml Calfthymus DNA) and incubated at 45°C for 2 hours to effect hybridization. Using Random Labelling Kit (Boeringer Mannheim), 20 - 50 ng of cDNA fragment of the protein of the present invention was labelled with [α-^{32}P.dDNA (0.74 MBq, Amersham) to make a probe. Into Hybripack were placed 3,000,000 cpm of the probe, 10 ml of the hybridization solution and the filter with sealing and they were incubated overnight at 45°C. After hybridization reaction, this was washed in 2 × SSC / 1% SDS at 45°C for 10 minutes. The washing was repeated twice and this was rinsed with 2 × SSC for a short time and then exposed to an imaging plate for 4 hours followed by analysis using BAS 2,000 (Fuji Film). Portions of 5 mm square of the plate corresponding to the sites where signal was detected were cut out and dipped in 500 μl of SM buffer to dissolve out the phage. Using this phage suspension, several hundreds pfu per plate of the phage was scattered and this was incubated at 37°C for 8 hours. Filters were prepared as above from 2 plates per a signal and hybridization was carried out using cDNA fragment for the protein of the present invention labelled with [α-^{32}P.dDNA] as the probe. Phages were recovered from regions where the signals were detected by an image analyzer and the third screening was conducted in the same manner as above to isolate 2 phage clones expected to have the gene for the protein of the present invention. The following analysis was conducted for one of the above two.

Step 3: Subcloning and sequencing of the gene for the protein of the present invention

(1) Preparation of phage DNA

Into 1 liter Erlenmeyer's flask NZYM medium was placed (0.2% maltose was added to NZY liquid medium). To this Escherichia coli XL1-Blue MRA (P2) (manufactured by Stratagene), cultured overnight with shaking at 37°C, was added and the mixture was cultured overnight with shaking at 37°C. When OD_{600} was 0.1, 5 × 10^{10} of the phage was added and the culture was continued. The value of OD_{600} was measured every hour and after lysis of *Escherichia coli* as the host was confirmed, 1.7 ml of chloroform was added and the mixture was stirred for 10 minutes. Phage DNA was prepared from 360 ml of the phage solution prepared in this manner using Lambda-trap (Clontech) to give about 21 g of the phage DNA.

(2) Characterization of the gene fragment for the protein of the present invention by Southern hybridization

With each 0.5 U of restriction enzymes NotI, XbaI and SalI, respectively, a 50 ng portion of the phage DNA obtained in (1) was digested and the product was electrophoresed on 0.8% agarose gel to fractionate DNA fractions. After checking migration length of DNA in the gel by ethidium bromide staining, the gel was rinsed for a short time and shaken in 0.25 N HCl for 15 minutes. Then the gel was rinsed again with water for a short time and shaken in 1.5 N NaCl/0.5 N NaOH for 30 minutes. The gel was blotted to a Nylon filter Hybond-N (Amersham) (in 1.5 N NaCl/0.5 N NaOH, at 55 Atm for 1 hour) using Vacugene (Pharmacia). The blotted filter was washed with 2 × SSC for 5 minutes, air-dried and irradiated for 2 minutes to fix the DNA onto the filter. The filter was packed with sealing in Hybripack together with the hybridization solution and incubated at 45°C for 2 hours. In Hybripack were packed with sealing 3,000,000 cpm of the cDNA probe for the protein of the present invention prepared as described in Step 2, (2), 10 ml of the hybridization solution and filter and they were incubated overnight at 45°C. After the hybridization reaction, the filter was twice washed in 2 × SSC / 1% SDS at 45°C for 10 minutes, rinsed with 2 ×SSC for a short time, exposed to an imaging plate for 2 hours and analyzed by BAS 2,000 (Fuji Film). As the result, it was demonstrated that the DNA fragment of 1.5 kb obtained by XbaI contained the gene for the protein of the present invention.

(3) Cloning of the DNA fragment containing the gene for the protein of the present invention

After completely digesting 2 μg of pBluescript KS-vector (Stratagene) with 10 U of XbaI, the terminal of the product was de-phosphorylated with CIAP (Takara Shuzo). To the reaction solution an equal amount of phenol/chloroform/isoamyl alcohol (25 : 24 : 1) was added and they were sufficiently mixed, followed by centrifugation. The upper layer was separated, combined with 1/10 amount of 3M sodium acetate and twice the amount of 100% ethanol and mixed well. The mixture was centrifuged after cooling at -80°C for 10 minutes. The obtained precipitates were rinsed with 70% ethanol, dried for a short time and suspended in TE. A 1 μg portion of phage DNA obtained in (1) was completely digested with 100 U of XbaI and the product was ligated to a 50 ng portion of pBluescript KS- vector prepared as above using a ligation kit (Takara Shuzo). An aliquot of the reaction solution was added to competent cells of *Escherichia coli* JM109 (Toyobo), transformed and the solution was spread on an LB plate containing 50 μg/ml of Ampicillin. After incubating overnight at 37°C, colony hybridization was carried out. Preparation of a filter, preparation of a probe, hybridization and washing were conducted in similar methods to those in (1) and (2), with the exception that hybridization and washing

temperature was 65°C. As the result of analysis by an image analyzer, many positive clones were obtained. From 18 clones among them, plasmid DNAs were prepared using QIA-prep spin column (Qiagen). The plasmid DNAs were digested with restriction enzyme XbaI which is suitable for cutting out the cloned DNA fragments and the product was fractionated with 0.8% agarose gel, analyzed and a clone, pCR16G1/Xb (see Fig. 2), containing a gene fragment (1.5 kb) of the protein of the present invention was selected. The selected clone was sequenced for the total nucleotide sequence using Taq Dye Deoxy Terminator Cycle Sequencing Kit (ABI) and a fluorescence sequencer (ABI) (see SEQ ID NOs: 1, 4 and 5). As the result, it was demonstrated that the clone contained a 247 bp promoter region (the promoter of the present invention), a 592 bp coding region (the gene of the protein of the present invention) and a 836 terminator region (the terminator of the present invention).

Example 2: Homology of the protein of the present invention

The nucleotide sequence of the gene of the protein of the present invention as determined in Example 1 (see SEQ ID NO:4) and the amino acid sequence of the protein of the present invention encoded by it (see SEQ ID NO: 3) were searched in data bases EMBL and NBRF. As the result, it was revealed that the gene of the protein of the present invention and the amino acid sequence encoded by it had a high homology with pollen allergen proteins in celery, white birch and the like, PR proteins associated with resistance to pathogen in parsley, potato and the like, proteins induced by stress from outer world in pea, soybean and the like (see Fig. 3). The highest homology was seen in APIg1 in celery, PR1-3 and PR1-1 which are PR proteins in parsley, contained in Umbelliferae plants which include carrot. Also, a relatively high homology was observed with mRNA of HSP60 which is a mouse heat shock protein.

Example 3: Analysis of expression pattern of the gene of the protein of the present invention by Northern hybridization

Total RNAs were extracted from each 2 g of flower, leaf and root of carrot, respectively, using Isogen (Nippon Gene), and further, about 40 μg of mRNA was prepared using Oligotex-dT30 (Takara Shuzo). A 5 μg portion of mRNA was fractionated with 1.2% modified agarose gel electrophoresis and blotted to a Nylon filter Hybond-N (Amersham) in 10 × SSC by the capillary blotting method. After blotting, the filter was air-dried and baked at 80°C for 2 hours to fix the DNA. The filter was dipped in a prehybridization solution and incubated at 45°C for 2 hours. Using pCR16G1/Xb as a template and a primer that amplifies 3'-non-coding region which has the follow sequence:
5'-GCTGA ACTTT CCACC GTGTT-3' and
5'-GACAT CTCAT AGTTG AGACT C-3',
PCR reaction (30 cycles in which 1 cycle consisted of treatments: 94°C, 1 minute; 55°C, 1 minute; 72°C, 1 minute) was conducted. In this reaction, a label was introduced by adding [α-^{32}P.dCTP], and using the product as the probe, the hybridization was conducted in a manner similar to that in Step 3, (2). The product, was analyzed by an image analyzer and it was confirmed that the gene for the protein of the present invention was highly transcribed in root.

Example 4: Obtaining the promoter of the present invention

Using a ligation kit (Takara Shuzo), 50 ng of pBluescript KS- vector completely digested with XhoI and dephosphorylated at the terminal was ligated with 1 μg of a phage genomic clone DNA completely digested with XhoI. An aliquot of the reaction solution was added to 100 μl of competent cells of *Escherichia coli* JM109 (Toyobo), transformed and the solution was spread on an LB plate containing 100 μg/ml of Ampicillin. After incubating overnight at 37°C, a plasmid was prepared from the growing clone using QIA-prep spin (Qiagen) and a region of several hundreds bp at the terminal of contained insert DNA was sequenced. As the result, pCR16G1/Xb (see Fig. 4), which is a clone containing a part of the coding region of the gene of the protein of the present invention and a 13 kb upstream region was obtained. The promoter region (2 kb) of the gene of the protein of the present invention contained therein was sequenced using TAq Dye Deoxy Terminator Cycle Sequencing Kit (ABI) and a fluorescence sequencer (ABI) (see SEQ ID NO: 1).

Example 5: Construction of the plasmid of the present invention

(1) Preparation of the promoter (247 bp) of the present invention

Using a sequence located inside the vector and a sequence located several tens bps upstream of ATG of the gene of the protein of the present invention as primers, which have the following sequences:
5'-GTAAA ACGAC GGCCA GT-3' (Made by Takara Shuzo) and
5'-GGGCT AGCGA CCTTT AGAAT GTTTT TGC-3',
and pCR16G1/Xb as a template, PCR reaction (40 cycles in which 1 cycle consisted of treatments: 94°C, 1 minute; 40°C, 2 minutes; 72°C, 3 minutes) was conducted to amplify the DNA fragment containing the promoter of the present

invention (247 bp). As the upstream primer of the gene ATG of the protein of the present invention, a synthesized primer having a recognition site for the restriction enzyme NheI at the 5' terminal was used. To the reaction product an equal amount of chloroform/isoamyl alcohol (24 : 1) was added, the obtained solution was centrifuged and the upper layer was separated. To this 1/10 amount of 3M ammonium acetate was added and twice the amount of 100% ethanol and mixed. The mixture was centrifuged to give precipitates, which was completely hydrolyzed with restriction enzymes XbaI and NheI and then fractionated by 4% polyacrylamide electrophoresis. The gel was cut out such that a DNA fragment having the desired length was contained and the DNA fragment was harvested according to the method described in Sambrook, supra. Thus, the gel was finely cut and the sections were dipped in a extraction buffer, incubated at 37°C for 5 hours and gel sections were removed by centrifugation. The solution was combined with twice the amount of 100% ethanol and the mixture was cooled on ice. Precipitates obtained upon centrifugation was suspended in TE. To this were added 1/10 amount of 3M ammonium acetate and twice the amount of 100% ethanol and ethanol precipitation was carried out as above. The obtained precipitates were re-suspended in TE and subjected to 4% polyacrylamide electrophoresis to check the concentration of the DNA fragment, which was used for the construction of the plasmid of the present invention.

(2) <u>Construction of the plasmid (pCR16G1/250-GUS) of the present invention</u>

The promoter of the present invention (247 bp) prepared as described in Example was ligated to a binary vector pBI101 (Clontech) which had been cut with XbaI and treated with CIAP and the resulting was used to infect *Escherichia coli* JM109 competent cells to obtain transformants. Clones grown on an LB plate containing 50 μg/ml of kanamycin was selected. A plasmid DNA was prepared therefrom and cut with a restriction enzyme to give a candidate clone containing the promoter of the present invention (247 bp). The clone was sequenced using a synthetic primer of which sequence is the same as that located inside the coding region of GUS gene and which has the following sequence:
5'-TCACG GGTTG GGGTT TCTAC-3'.
It was confirmed that no nucleotide substitution has occurred in the 247 bp promoter region by Taq polymerase (see Fig. 5).

(3) <u>Construction of the (expression) plasmid (pCR16G1/EV-GUS) of the present invention</u>

pCR16G1/XhoI was cut by EcoRV and the resulting fragment was fractionated by 0.8% agarose gel electrophoresis. A band corresponding to a DNA fragment containing the promoter of the present invention (247 bp) was cut out and the DNA fragment was recovered using glass beads (Bio-Rad). Concentration of DNA in the obtained DNA fragment was checked by 0.8% agarose gel electrophoresis and the DNA was ligated to a binary vector pBI101 digested with a restriction enzyme SmaI. *Escherichia coli* JM109 competent cells were infected with the fragment to transform the cells. Clone grown on an LB plate containing 50 μg/ml of kanamycin was selected. A plasmid DNA was prepared from the grown clones and orientation of the insert was investigated by restriction enzyme digestion. A clone having an insert in normal orientation was taken. This clone was mass-cultured and the plasmid DNA was mass-produced using Qiagen Tip-500 column (Qiagen). The plasmid DNA was digested with XbaI, fractionated by 0.8% agarose gel electrophoresis and 1.75 kb DNA fragment (containing upstream region of the 247 bp) was recovered in a manner similar to that described above. This was treated with CIAP and ligated to pCR16G1/250-GUS that had been digested with a restriction enzyme XbaI (connection site of NheI and XbaI became uncleavable). The resulting fragment was used to transform *Escherichia coli* JM109 competent cells,
and a plasmid was prepared from a kanamycin resistant clone. By restriction digestion, it was confirmed that the clone contained the promoter of the present invention (2 kb). Further, using a synthetic primer having the same sequence as that located inside the GUS coding region, which have the following sequence:
5'-TCACG GGTTG GGGTT TCTAC-3',
the promoter region was sequenced and the structure of about 500 bp upstream of ATG in GUS gene was characterized (SEQ ID NO: 2, see Fig. 6).

(4) <u>Construction of the plasmid (pCR16G1/H-GUS) of the present invention</u>

pCR16G1/XhoI was cut by XbaI and a DNA fragment containing 5 kb sequence located upstream of the gene ATG of the protein of the present invention was recovered in the same manner as (3) above. The resulting fragment was ligated to a binary vector pBI101 that had been digested with a restriction enzyme XbaI. The product was used to transform competent cells. A clone having an insert in normal orientation was obtained. This clone was further digested with HindIII, self-ligated, transformed and it was confirmed that the obtained clone had only one HindIII restriction site. The plasmid DNA was digested with XbaI and ligated to the promoter of the present invention (247 bp) as described above. A plasmid was prepared using this clone having the promoter of the present invention (247 bp) inserted in the normal

orientation. Using a synthetic primer having the same sequence as that located inside the GUS coding region, which has the following sequence:

5'-TCACG GGTTG GGGTT TCTAC-3',

the plasmid was sequenced in the same manner as above and the structure of about 500 bp upstream of ATG in GUS gene was characterized (see Figs. 7 and 8). This allowed to confirm that the structure at and around the ligation site was not changed.

Example 6: Production of cells and a plant of the present invention

Production of a transgenic plant was performed according to the method described in S. B. Gelvin, R. A. Schileroort and D. P. S. Verma, Plant Molecular Biology/Manual (1988) (Kliwer Academic Publishers; Valvekens et al., Proc. Natl. Acad. Sci., 85, 5536 - 5540 (1988)).

Agrobacterium LBA4404 strain cultured overnight in YEB medium at 30°C with shaking was subcultured to a fresh YEB medium and the cultivation was continued until OD_{600} was 0.6. The following procedure was conducted in a cooled room. Cells were collected from the culture by centrifugation, suspended in cooled distilled sterile water and collected by centrifuging again. This washing operation was twice repeated and a similar operation was performed replacing distilled sterile water by 10% glycerol solution. The obtained cells were suspended in 10% glycerol so as to obtain finally at 400 times the concentration. To these competent cells the three Ti plasmid expression vectors (hereinafter referred to as pCR16G1/250-GUS, pCR16G1/EV-GUS and pCR16G1/H-GUS) constructed as described above were introduced by electroporation method and selected on YEB plate containing 50 μg/ml of kanamycin. Plasmid DNAs were prepared from grown kanamycin-resistant clones by alkali-SDS method, and it was confirmed by 0.8% agarose gel electrophoresis and ethidium bromide staining that the Ti expression vectors were introduced. The strains of *Agrobacterium* (pCR16G1/250-GUS/LBA4404, pCR16G1/EV-GUS/LBA4404 and pCR16G1/H-GUS/LBA4404) were cultured with shaking in YEB liquid medium at 30°C over 2 nights.

After non-symbiotic seeding, roots of *Arabidopsis* grown at 23°C for 2 - 3 weeks that were cut into about 1 cm were incubated on CIM plates for 2 days, dipped in a culture of *Agrobacterium* (pCR16G1/250-GUS/LBA4404, pCR16G1/EV-GUS/LBA4404 or pCR16G1/H-GUS/LBA4404) cultivated with shaking at 30°C over 2 nights and incubated again on CIM plates. After 2 days, the sections of root were transferred to SIMC medium and, further 2 days later, they were subcultured to SIMCK medium. About 1 month after, regenerated shoot was cut off and transplanted to RIM medium to cause rooting. A rooted individuals were planted in soil or rock wool, grown in an air-conditioned room to obtain self-propagated seeds.

Sections of aseptically incubated tobacco leaves were dipped in MS medium. Aliquot of 30°C-overnight culture of *Agrobacterium* strains (pCR16G1/250-GUS/LBA4404, pCR16G1/EV-GUS/LBA4404, pCR16G1/H-GUS/LBA4404) was added to the medium and co-incubation was continued at 25°C for 2 days in the dark. Thereafter, the sections of leaves were washed with MS liquid medium and placed on MS-NBCK medium. They were stationarily cultured at 25°C for about 1 month in the light, a regenerated shoot was cut off from the section of leaf and transplanted to MS-CK medium. About 1 month later, rooted individuals were planted in soil, grown in a green house and self-propagated seeds were obtained.

Example 7: Detection of the presence of the introduced gene in the plant of the present invention

The seeds of transgenic *Arabidopsis* obtained in Example 6 were sterilized with 1% hypochlorous acid for 5 minutes, washed 3 - 5 times with sterilized distilled water and then non-symbiotically germinated in MS medium containing 20 μ g/ml kanamycin. From an individual exhibiting kanamycin resistance, 4 - 5 rosette leaves were taken and the genomic DNA was prepared from them by CTAB method.

Using 50 ng of this DNA as a template and a sequence located inside the GUS gene which is a reporter gene and a sequence located around 250 bp upstream of ATG in GUS gene (inside the promoter of the present invention) as primers, which have following sequences:

5'-TCTGC ATCGG CGAAC TGATC-3' and

5'-ACAAA CACAG CACTA ACTTT TC-3'

and further, using a sequence located inside GUS gene and a sequence located inside NOS terminator as primers, which have the following sequences:

5'-ACATG TGGAG TGAAG AGTAT C-3' and

5'-CATGC TTAAC GTAAT TCAAC AG-3',

PCR reactions (40 cycles in which 1 cycle consisted of treatments: 94°C, 1 minute; 55°C, 2 minutes; 72°C, 3 minutes) were conducted, then a portion of PCR product was fractionated by 0.8% agarose gel electorphoresis. Thus, the presence of the introduced gene was confirmed by the amplification of the desired DNA fragment.

Also, for the transgenic tobacco, the seeds were sterilized with 2.5% hypochlorous acid/0.002% Triton X-100 for 5

minutes, washed 4 - 5 times with sterilized water. The seeds were non-symbiotically germinated in MS medium containing 100 μg/ml kanamycin. Using an individual among those exhibiting kanamycin resistance, the genomic DNA was prepared by CTAB method: the same method as that for *Arabidopsis*, i. e. using an individual among those exhibiting kanamycin resistance, the genomic DNA was prepared by CTAB method. Using 50 ng of this DNA fragment as a template and a sequence located inside the GUS gene and a sequence located around 250 bp upstream of ATG in GUS gene (inside the promoter of the present invention), which have the following sequences:
5'-TCTGC ATCGG CGAAC TGATC-3' and
5'-ACAAA CACAG CACTA ACTTT TC-3'
and further, using a sequence located inside the GUS gene and a sequence located inside NOS terminator as primers, which have following sequences:
5'-ACATG TGGAG TGAAG AGTAT C-3' and
5'-CATGC TTAAC GTAAT TCAAC AG-3',
PCR reactions (40 cycles in which 1 cycle consisted of treatments: 94°C, 1 minute; 55°C, 2 minutes; 72°C, 3 minutes) were conducted, then a portion of PCR product was fractionated by 0.8% agarose gel electorphoresis and the presence of the introduced gene was confirmed by the amplification of the desired DNA fragment.

Example 8: Confirmation of expression pattern of the introduced gene

Measurement of GUS staining and GUS activity in the leaf and root of seeding from the plant of the present invention (containing the plasmids of the present invention:
pCR16G1/250-GUS, pCR16G1/EV-GUS, pCR16G1/H-GUS and, as controls, pBI121 (manufactured by Clontech), pBI101) obtained in Example 6 was conducted according to the method described in Plant Mol. Bio. Rep., 5, 387 - 405 (1987).

The measurement of GUS activity was carried out by the fluorescence method using 4-methylumbelliferyl-glucuronic acid as the substrate and that of activity staining was carried out by determining deposition of a blue pigment (indigotin) using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) as the substrate.

(1) GUS staining

The seeds of transgenic Arabidopsis were non-symbiotically germinated in MS medium containing 20 μg/ml kanamycin and individuals exhibiting kanamycin resistance were grown for 3 weeks. The plants were pulled out taking care not to damage the root, dipped in GUS staining solution (1 mM X-Gluc, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $Fe_4Fe(CN)_6$, 0.3% Triton X-100) and incubated overnight at 37°C. After the reaction was completed, they were decolorized by washing several times with 100% ethanol and staining pattern was observed. The results confirmed that the product of the introduced gene was highly expressed in vascular bundles, particularly in vascular bundles of the root, in every plant of the present invention (see Figs. 9 and 10). The intensity of expression was the strongest in the plant of the present invention in which pCR16G1/H-GUS was introduced (see Table 1).

The seeds of transgenic tobacco were non-symbiotically germinated in MS medium containing 100 μg/ml kanamycin and individuals exhibiting kanamycin resistance were pulled out after 1 or 3 weeks or 1 month. They were dipped in GUS staining solution and incubated overnight at 37°C. Then, they were decolorized with 100% ethanol and staining pattern was observed. The results confirmed the similar tendency to that of the expression in transgenic *Arabidopsis*. It was also confirmed that the staining became stronger with the progress of growth stage.

(2) Measurement of GUS activity

The seeds of transgenic tobacco were non-symbiotically germinated in MS medium containing 100 μg/ml kanamycin and incubated at 25°C for 1 month. To 0.8 g of root and 0.5 g of leaves respectively placed in a mortar was added 1 ml or 0.5 ml, respectively, of an extraction buffer (50 mM phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% Sarcosyl, 10 mM mercaptoethanol) and they were triturated with an appropriate amount of sea sand. The triturate was transferred to an Eppendorff tube, centrifuged and the supernatant was taken out. Aliquots of 10 - 70 μl were added to 500 ml of a reaction substrate solution (50 mM phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% Sarcosyl, 10 mM mercaptoethanol, 1 mM 4-methylumbelliferyl-β-D-glucuronide) and reacted at 37°C. After reacting, 100 μl aliquot samples were taken out and immediately mixed with 900 μl reaction quenching solution (0.2 M sodium carbonate solution). The treated samples were assayed by a spectrophotofluorometer (model F-2000, Hitachi Seisakusho). GUS activity was calculated from the results of measurement and protein concentrations in extracts from leaves and root. Determination of protein concentration was carried out by a method using Protein Assay Reagent available from Bio-Rad. As the result, the highest activity was detected in the root, in the plant of the present invention (see Table 2).

Table 1

Comparison of GUS activity in root of transgenic tobacco plants into which various promoters were introduced:	
Introduced Gene	Ratio of GUS Activity
pCR16G1/250-GUS	121
pCR16G1/EV-GUS	401
pCR16G1/H-GUS	305
PBI101 (no promoter-GUS)	100
* The ratio is calculated taking GUS activity in root of pBI101 as 100.	

Table 2

Comparison of GUS activity in various tissues of transgenic tobacco plants into which various promoters were introduced:		
Introduced Gene	Ratio of GUS Activity	
	Leaf	Root
pCR16G1/250-GUS	1	56
pCR16G1/EV-GUS	1	34
pCR16G1/H-GUS	1	245
pBI121 (35S promoter-GUS)	1	4
* The ratio is calculated taking GUS activity in leaf as 1.		

Composition of media used in Examples are shown below:

(1) Media for tobacco plants

a) MS agar medium

Into 1 liter of distilled water 4.4 g of Murashige and Skoog (Flow Laboratories) and 30 g of sugar were dissolved.

The solution was adjusted to pH 5.8 with 1 M KOH, combined with 3 g of gellan gum (Wako Pure Chemical) and sterilized in an autoclave.

b) MS-NBCK agar medium

This medium was prepared by adding 0.1 μl/mg of 1-naphthaleneacetic acid (NAA), 1.0 μg/ml of 6-benzylaminopurine (BA), 20 μg/ml of kanamycin and 300 μg/ml of claforan to MS agar medium.

c) MS-CK agar medium

This medium was prepared by adding 100 μg/ml of kanamycin and 300 μg/ml of claforan to MS agar medium.

(2) Media for *Arabidopsis*

a) MS agar medium

Into 1 liter of distilled water 4.4 g of Murashige and Skoog (Flow Laboratories) and 20 g of sugar were dissolved.

The solution was adjusted to pH 6.3 with 1M KOH, combined with 2 g of gellan gum (Wako Pure Chemical) and sterilized in an autoclave.

b) CIM agar medium

This medium was prepared by adding 0.5 μg/ml of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.05 μg/ml of kinetin to MS agar medium.

c) SIMC agar medium

This medium was prepared by adding 5 μl/mg of [2-isopentenyl.adenine (2-Pi), 0.15 μg/ml of indolacetic acid (IAA) and 300 μg/ml of claforan to MS agar medium.

d) SIMCK agar medium

This medium was prepared by adding 20 μl/mg of kanamycin to SIMC medium.

(3) Media for bacteria and phages

a) L medium

Into 1 liter of distilled water 10 g of Bactotrypton (Difco), 5 g of Bacto yeast extract (Difco) and 10 g of Nacl were dissolved.

The solution is adjusted to pH 7.0 with 5 M NaOH and sterilized in an autoclave. For plate medium, 15 g of agar is added.

b) YEB medium

Into 1 liter of distilled water 5 g of Bacto beef extract (Difco), 1 g of Bacto yeast extract (Difco), 5 g of polypeptone, 5 g of sugar and 0.2 ml of 10 M NaOH were dissolved. The solution is sterilized in an autoclave. Thereafter, 0.2 ml of filter-sterilized 1M $MgSO_4$ is added on use. For plate medium, 15 g of agar is added.

c) NZY medium

Into 1 liter of distilled water 5 g of yeast extract, 10 g of NZ amine, 5 g of NaCl and 2 g of $MgSO_4 \cdot 7H_2O$ were dissolved.

The solution is adjusted to pH 7.5 with 5M NaOH and sterilized in an autoclave. For plate medium, 15 g of agar (Difco) is added.

d) Top agar

This medium is prepared by adding 0.7 g of Agarose-II (Dojin) to 100 ml of NZY medium.

EFFECT OF THE INVENTION.

By utilizing the promoter of the present invention, high expression of a desired protein in plant vascular bundles (particularly in vascular bundles in root) became possible.

SEQUENCE LISTING

SEQ ID NO: 1

SEQUENCE LENGTH: 247

SEQUENCE TYPE: Nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: genomic DNA

FEATURES OF SEQUENCE

ORIGINAL SOURCE

ORGANISM: carrot (*Daucus carota L.*)

CALTIVAR: Kuroda Gosun

KEY: promoter

LOCATION: 1..247

SEQUENCE DESCRIPTION

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TTCTAGAATA TATCTTTTGA AATTTCAACA AACACAGCAC TAACTTTTCT TTTAACAGAT   60
TAGAATCGTT TCCTAAACTT TTAAAATTAA AAAATACATT ACTATAATAT TTATCAACAC  120
CTCAACATTC ATGTTAGCGT ACTATAAATA GGTGCTCTTG GTGCTCTACT ATCATCACAT  180
CAATCTTCCA GCACAAACCT TGAGCTTAAT CTTTCTACTA ATTTTTAGCA AAAACATTCT  240
AAAGGTC                                                            247
```

SEQ ID NO: 2

SEQUENCE LENGTH: 2042

SEQUENCE TYPE: Nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: genomic DNA

FEATURES OF SEQUENCE

ORIGINAL SOURCE

ORGANISM: carrot (*Daucus carota L.*)

CALTIVAR: Kuroda Gosun

KEY: promoter

LOCATION: 1..2042

SEQUENCE DESCRIPTION

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TCTCGGGCCA CTTTCAAGGA TAAAAGCACC GGGAAGAACG AGAAATCCGT AGTTCCGTGG   60
AATTGAGATC TAAGAAAAGA AGGCCAAATC GGAAAAGGTT TTGAATCCTT AGATCCGGAA  120
AAACAAATGC CAAAGAAGTT TTATTGCAAG AAAAAAGCAA ACACAAAAGA AGAGAAATAA  180
AAAATTTTGG GGCTTTCCAC CGGTAATGGA AGAATATGCA CAAAAATTCA CGCCAACAGA  240
GTCCTTACTT AACTCTCACC TTTTGCACAC TCTTTCTCAT ATTTTTTTTT ATCTTTTTGT  300
TCAAAAAATT TGAATATAAT ATAATAATAA TAATAATAAT AATAATAATA ATAATAATAA  360
TAATAATAAT AATAATAATA ATAATAATAA TAATAATAAT AGGGAAGGCA AATAGGGATC  420
ATTGTTCGAG TTGTCAATCA ATACGGAGTC AATTGAAGTG TTATATATTT AGGATACCTT  480
CTTATTACAC AGCTGGAGAT GTTCTAGTCT ATCGAACTTA AAATTCCTCC AAATACAAAA  540
TATTTCTTAT GAAGAGCATC AACAGAATAA TTTCCAACTA ACACCCAATC GAGAAAGAGA  600
TTGATGCTTA TTGCCCAGTT TGTAATGCTG AAGCAGAGAC TACTCTTCAT GCGTTCGTTA  660
CACCTCATCA GTTCGCTAAT TACAAGACTT ATTGGGATAG TGTTGAGAGT CTAATTACAG  720
CTACAGAGCA TGCTTCCTTT TTAGAATGGT TGAGCAATAC TTTCAACCAG GTGAAGAGTT  780
AAATCGGAGG GTAATGCTAA GTTGGGCCCT ATGGAAGAAC TGAAATGAGT TAGTGTGGCA  840
CCAAAGTATT ATGGAAATTA CAGGGGTGAC ATGTCTGCAC AACGGGCCCT TATACAACAC  900
TTTGGGCATG TTTGGGAAAG ACAGCTTATG GCTTTTTTTA TAAAGAGTCA GCTTCTACTT  960
CTCTTGACCC GTTTGTGTAA AAGGTTAGAA GCACTTAAAA AAAACCGACA ATACTAACTT 1020
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TAGTTTCAAG ACTTCTGCTT CTTTCCCAAA CAATTTAATC ACTTATAAAT CTTAATTTAC 1080

TTCTTACTTC TGGTGCACTT CTTTACTTTA TGCAAGAGAC ACTTTTTTTA AGTTTAACCA 1140

AACGACCCTT TCTCATCCCT TGTTCGAGTA GTCGAAGAAT GCAAAGAGAA GTAAGAATCA 1200

GCAGGTGTCA CTACAGTTTG CAAAATGACA CGCAAATAAA GTAGCCCACC GCTCAGTGAG 1260

ATATTGATTC TACCATTGAT CGTGTTTGGT GTGTAGATGA TGCACACATG GACTTCATTC 1320

ACGTAATGTT GAACGATTTG ATAAATTAGT GAAATTTCAT TTCTTGGGCA AAAAAAGTCC 1380

CAAAGTCTAT ATAGGTTCTA AGTGAAACCA ACTCCTAAAT TATACAGCTA AATTGAGCAT 1440

CAGTGGAATC CATCTTCTCA ATTATAAATG CAAATAGAAT TAGTACATAT AACTAGAATT 1500

TAAATTAACA TATGTAATTC ATGTAACGGT CTACATCGCA TGAAATTATT TATCTGAATG 1560

ATAACATCTT TGTAAACAAA ACTGGGCCAA ATAGGACCAT AACCAAGTTC ACGTGTATTC 1620

TAAAATGTTA ATACTAACAT GAGTATTTTC TTTTCAAGGT ATAAGTTAAT TCTTCAATCA 1680

ATTAACTTTA AATTTGGACA TTATTGAGCA ACTTTATGCC CACGTTGTAT TGTTTAAACA 1740

ACGTTTGTCC GGTGTATATT TATGACCTTT CAACTCAAGC TAGCCAGTGA ATGCTTTCTA 1800

GAATATATCT TTTGAAATTT CAACAAACAC AGCACTAACT TTTCTTTTAA CAGATTAGAA 1860

TCGTTTCCTA AACTTTTAAA ATTAAAAAAT ACATTACTAT AATATTTATC AACACCTCAA 1920

CATTCATGTT AGCGTACTAT AAATAGGTGC TCTTGGTGCT CTACTATCAT CACATCAATC 1980

TTCCAGCACA AACCTTGAGC TTAATCTTTC TACTAATTTT TAGCAAAAAC ATTCTAAAGG 2040

TC 2042

SEQ ID NO: 3

SEQUENCE LENGTH: 157

SEQUENCE TYPE: Amino acid

TOPOLOGY: linear

MOLECULE TYPE: protein

FEATURES OF SEQUENCE

ORIGINAL SOURCE

ORGANISM: carrot (*Daucus carota L.*)

CALTIVAR: Kuroda Gosun

KEY: peptide

LOCATION: 1..154

SEQUENCE DESCRIPTION

```
 1               5                   10                  15
Met Gly Ala Gln Ser His Ser Leu Glu Ile Thr Ser Ser Val Ser Ala
                20                  25                  30
Glu Lys Ile Phe Ser Gly Ile Val Leu Asp Val Asp Thr Val Ile Pro
            35                  40                  45
Lys Ala Ala Pro Gly Ala Tyr Lys Ser Val Asp Val Lys Gly Asp Gly
        50                  55                  60
Gly Ala Gly Thr Val Arg Ile Ile Thr Leu Pro Glu Gly Ser Pro Ile
65                  70                  75                  80
Thr Ser Met Thr Val Arg Thr Asp Ala Val Asn Lys Glu Ala Leu Thr
                85                  90                  95
Tyr Asp Ser Thr Val Ile Asp Gly Asp Ile Leu Leu Gly Phe Ile Glu
            100                 105                 110
Ser Ile Glu Thr His Leu Val Val Val Pro Thr Ala Asp Gly Gly Ser
        115                 120                 125
Ile Thr Lys Thr Thr Ala Ile Phe His Thr Lys Gly Asp Ala Val Val
    130                 135                 140
Pro Glu Glu Asn Ile Lys Phe Ala Asp Ala Gln Asn Thr Ala Leu Phe
145                 150                 154
Lys Ala Ile Glu Ala Tyr Leu Ile Ala Asn
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SEQ ID NO: 4

SEQUENCE LENGTH: 593

SEQUENCE TYPE: Nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: genomic DNA

FEATURES OF SEQUENCE

ORIGINAL SOURCE

ORGANISM: carrot (*Daucus carota L.*)

CALTIVAR: Kuroda Gosun

KEY: CDS

LOCATION: 1..593

KEY: intron

LOCATION: 184..311

SEQUENCE DESCRIPTION

```
1               5                   10                  15
ATG GGT GCC CAG AGC CAT TCA CTC GAG ATC ACT TCT TCA GTC TCC GCA    48
            20                  25                  30
GAG AAA ATA TTC AGC GGC ATT GTC CTT GAT GTT GAT ACA GTT ATC CCC    96
        35                  40                  45
AAG GCT GCC CCT GGA GCT TAC AAG AGT GTC GAT GTT AAA GGA GAT GGT   144
    50                  55                  60
GGA GCT GGA ACC GTC AGA ATT ATC ACC CTT CCC GAA GGT               183
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TAGTTATATA TCTCACCCCA TCTTGTTGAT GTATCATTTC TGATACCATA TTAATTTGAG 243

GGGATTATTT CCCGACATTG TACAATTAAT AAATTTTTTG AATACATATA TAATTCTCTG 303

CTGCAGGT 311

65 70 75

AGC CCG ATC ACC TCA ATG ACG GTT AGA ACT GAT GCA GTC AAC AAG GAG 359

80 85 90

GCC TTG ACA TAC GAC TCC ACC GTT ATT GAT GGA GAC ATC CTT TTA GGC 407

95 100 105

TTC ATC GAA TCC ATT GAA ACC CAT CTT GTC GTT GTG CCA ACT GCT GAC 455

110 115 120 125

GGG GGT AGC ATT ACC AAG ACC ACG GCC ATA TTC CAC ACT AAA GGT GAT 503

130 135 140

GCC GTC GTT CCT GAA GAG AAC ATC AAG TTT GCA GAT GCT CAG AAC ACC 551

145 150 155

GCT CTC TTC AAA GCT ATC GAG GTC TAC CTC ATT GCT AAC TAA 593

SEQ ID NO: 5

SEQUENCE LENGTH: 836

SEQUENCE TYPE: Nucleic acid

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: genomic DNA

FEATURES OF SEQUENCE

ORIGINAL SOURCE

ORGANISM: carrot (*Daucus carota L.*)

CALTIVAR: Kuroda Gosun

KEY: terminator

LOCATION: 1..836

SEQUENCE DESCRIPTION

```
GCTGAACTTT CCACCGTGTT TTAATAATCT GTCGTTTTTA AATTTATGGG AAGAGCGCCA   60
AAGATGCCTC AACTTCATAA TTTTATGAGC GGGCGAtAGA ATTGCAACTT TTTCTTTGTA  120
CTCTGTTTTA ATGAGCAATT TCATTTGGTA ACAATATGTG TAATCTTTTT AAATAAAATA  180
TAGTACCGAC ATTAATGTAA TCTTTCTGGA TCATCTGTGC TTTCATATGT TACTTATATT  240
TTTTAGTTAA AAATGTAATT CACTTGAACC TTAATGATAT ATAGGTCATC CCAATTAATT  300
AATTTCAAGT TTCGGTTTGA AATTAGAAAG AGTAAAGAAT TTGTAGTATG AACGATGAGT  360
CGATGACAGA AAAAAGAAGC TTGCAGTGTC CCAAAAAGAT AAATTTAATT ATTTCATTAA  420
GTGAGAATGA TAAGACTCAG TAAACCTCCT CAGTTAGTCC ATCCAACCCT TATAAGCCTG  480
ATAACTGGTG ATTAATTGTA ATGATGTTTT ATTACTATGG GGCAGTTTGG CTGGACTTAA  540
AAAAAGTGAC TTATTGCTTA AAATAAATAA GTAGATTATA AGTGAAAAGT TGATTTGGAC  600
TTATAAGTTA TTAAAAGTGT TTGAATATAT ATTGATTATA AGTGATAGAA GAAGCTAAAT  660
CCCCAAAATA AGCTAGGTTT CCTAACTTCT TTTTTGGGGC TTTTAAGCTT CAATATAAGT  720
GCTTCTCATA ATTAGCCAAA CACCTCCATT TAAGTAGAAG TCGACTTCTA TGTTAAAAAA  780
GCTCCGAAGT CGGTTTGCCA AACACCCCCT ATATGGGTCT ATTCTTGGCA TCTAGA      836
```

Claims

1. A promoter which is functional in plant cells comprising a nucleotide sequence (about 250 bp) of SEQ ID NO: 1.

2. A promoter which is functional in plant cells comprising a nucleotide sequence (about 2 Kbp) of SEQ ID NO: 2.

3. A promoter which is functional in plant cells comprise a nucleotide sequence (bout 4 Kbp) having the following characteristics:

   a. isolated and/or purified from carrot;
   b. having restriction enzyme sites for XhoI (0 kb), XbaI (0,3 kb), EcoRV (2 kb), EcoRV (2.3 kb), EcoRI (3 kb), SmaI (3.6 kb) and HindIII (4 kb); and
   c. containing a nucleotide sequence of SEQ ID NO: 2.

4. A plasmid comprising a promoter as defined in claim 1.

5. A plasmid comprising a promoter as defined in claim 2.

6. A plasmid comprising a promoter as defined in claim 3.

7. A chimera gene comprising a promoter as defined in claim 1 and a desired structural gene.

8. A chimera gene comprising a promoter as defined in claim 2 and a desired structural gene.

9. A chimera gene comprising a promoter as defined in claim 3 and a desired structural gene.

10. A plasmid comprising a chimera gene as defined in claim 7.

11. A plasmid comprising a chimera gene as defined in claim 8.

12. A plasmid comprising a chimera gene as defined in claim 9.

13. A plasmid shown as in Fig. 1.

14. A microorganism containing the plasmid according to claim 4, 5 or 6.

15. A microorganism containing the chimera gene according to claim 7, 8 or 9.

16. A plant cell wherein the expression of the desired protein is under control of the promoter of claim 1, 2 or 3.

17. A plant cell containing the chimera gene of claim 7, 8 or 9.

18. A plant into which the promoter of claim 1, 2 or 3 has been introduced, wherein a desired protein is expressed.

19. A plant containing the chimera gene of claim 7, 8 or 9.

20. A process for preparing a chimera gene which comprises ligating a desired structural gene downstream of the promoter of claim 1, 2 or 3.

21. A protein having a molecular weight of 16 kD and having an amino acid sequence of SEQ ID NO: 3.

22. A gene coding for a protein having a molecular weight of 16 kD, having a nucleotide sequence encoding an amino acid sequence of SEQ ID NO: 3.

23. A gene coding for a protein having a molecular weight of 16 kD, having a nucleotide sequence of SEQ ID NO: 4.

24. A plasmid comprising the gene according to claim 23.

25. A terminator which is functional in plant cells comprising a nucleotide sequence of SEQ ID NO: 5.

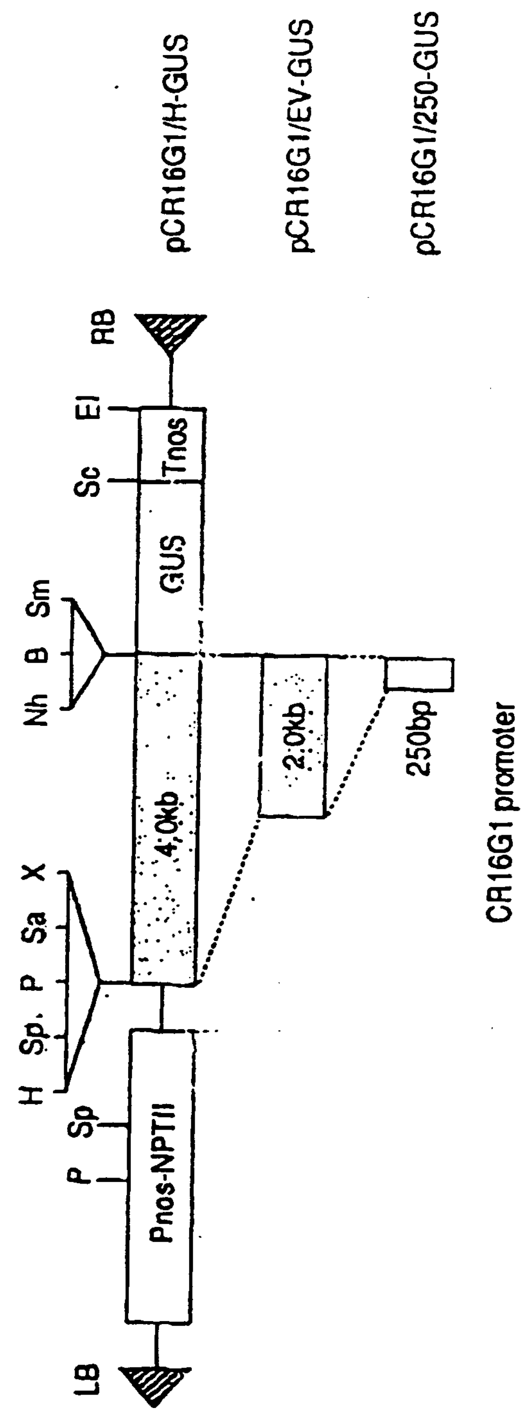

LB
Pnos-NPTII
P
Sp
H
Sp.
P
Sa
X
4,0kb
2,0kb
250bp
Nh
B
Sm
GUS
Sc
Tnos
El
RB
pCR16G1/H-GUS
pCR16G1/EV-GUS
pCR16G1/250-GUS
CR16G1 promoter

Fig.1

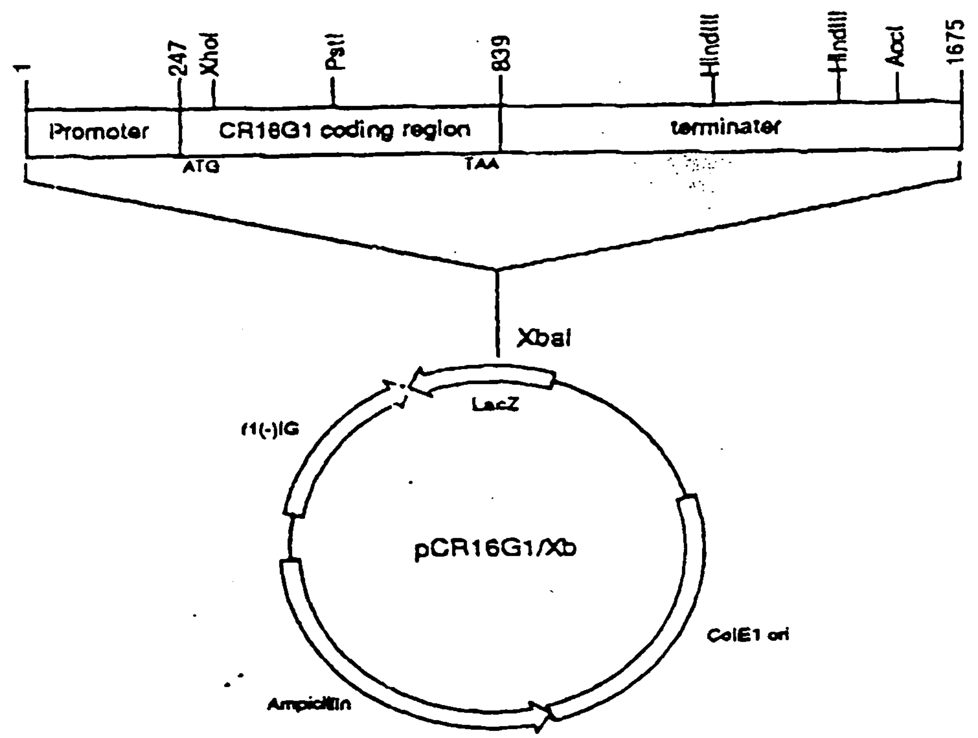

1
247
XhoI
PstI
839
HindIII
HindIII
AccI
1675
Promoter
CR16G1 coding region
terminater
ATG
TAA
XbaI
LacZ
f1(-)IG
pCR16G1/Xb
ColE1 ori
Ampicillin

Fig.2

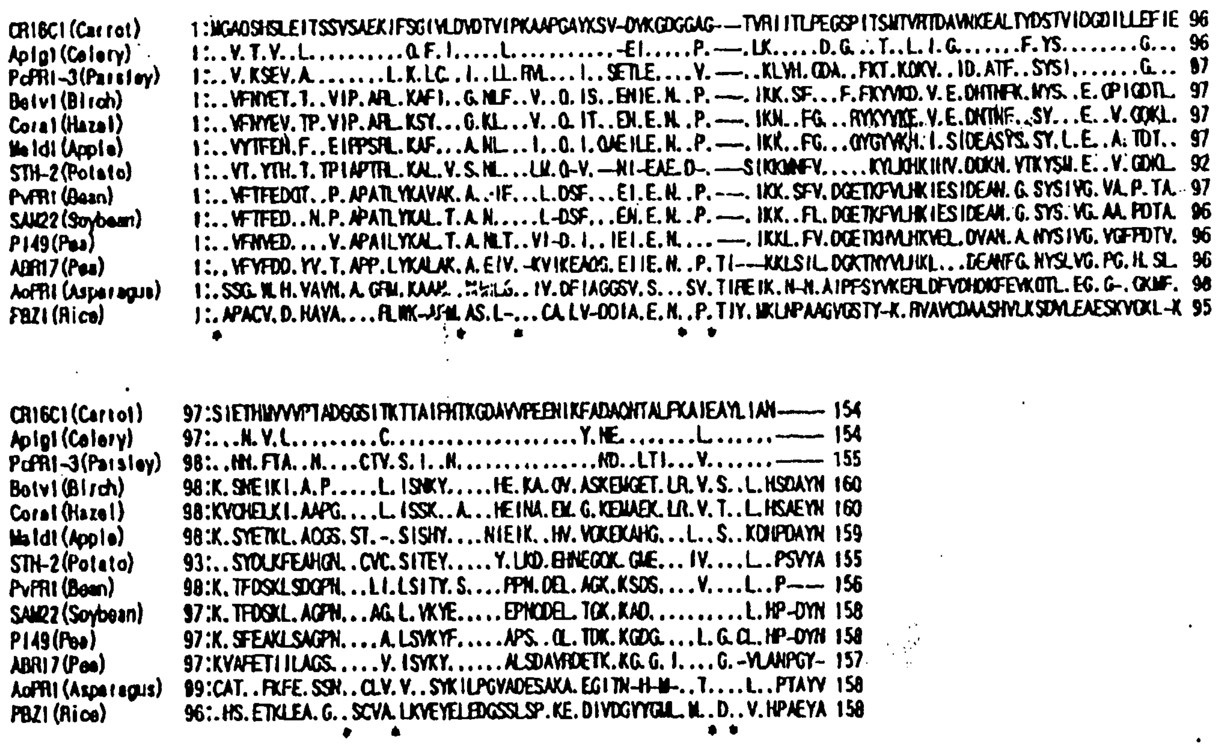

CR16C1 (Carrot)
Apig1 (Celery)
PcPR1-3 (Parsley)
Betv1 (Birch)
Cora1 (Hazel)
Mald1 (Apple)
STH-2 (Potato)
PvPR1 (Bean)
SAM22 (Soybean)
P149 (Pea)
ABR17 (Pea)
AoPR1 (Asparagus)
PBZ1 (Rice)

Fig. 3

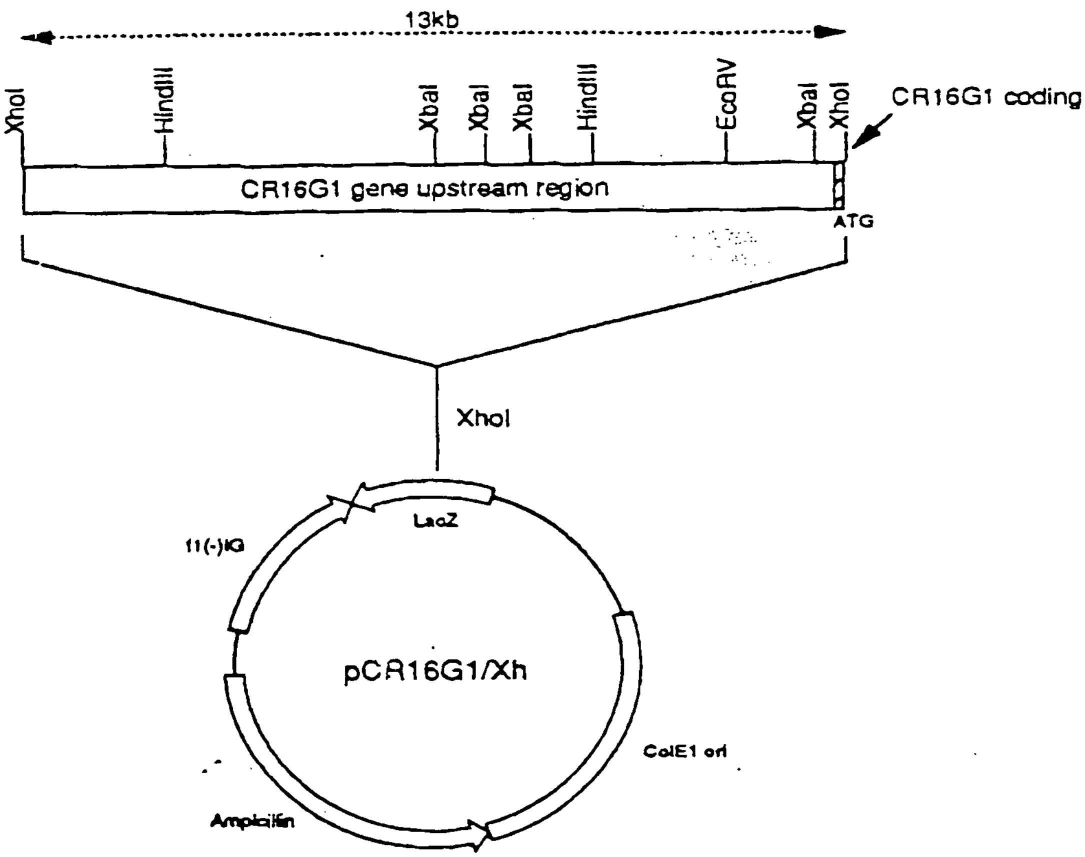

13kb
XhoI
HindIII
XbaI
XbaI
XbaI
HindIII
EcoRV
XbaI
XhoI
CR16G1 coding
CR16G1 gene upstream region
ATG
XhoI
LacZ
f1(-)IG
pCR16G1/Xh
ColE1 ori
Ampicillin

Fig.4

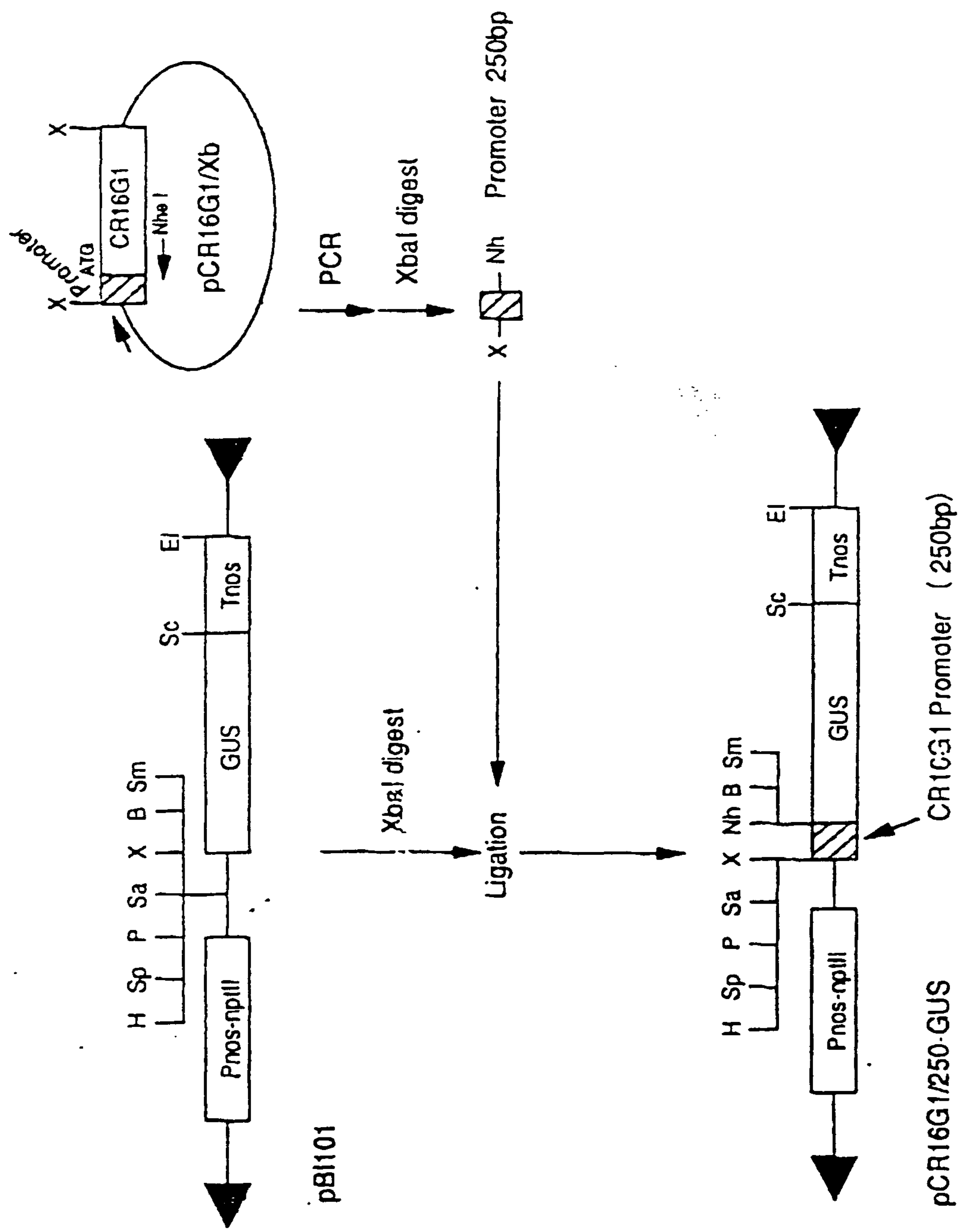

pCR16G1/Xb
CR16G1
Promoter
ATG
Nhe I
X
PCR
XbaI digest
X
Nh
Promoter 250bp
H Sp P Sa X B Sm
Pnos-nptII
GUS
Sc
Tnos
EI
pBI101
XbaI digest
Ligation
H Sp P Sa X Nh B Sm
Pnos-nptII
GUS
Sc
Tnos
EI
CR16G1 Promoter (250bp)
pCR16G1/250-GUS

Fig.5

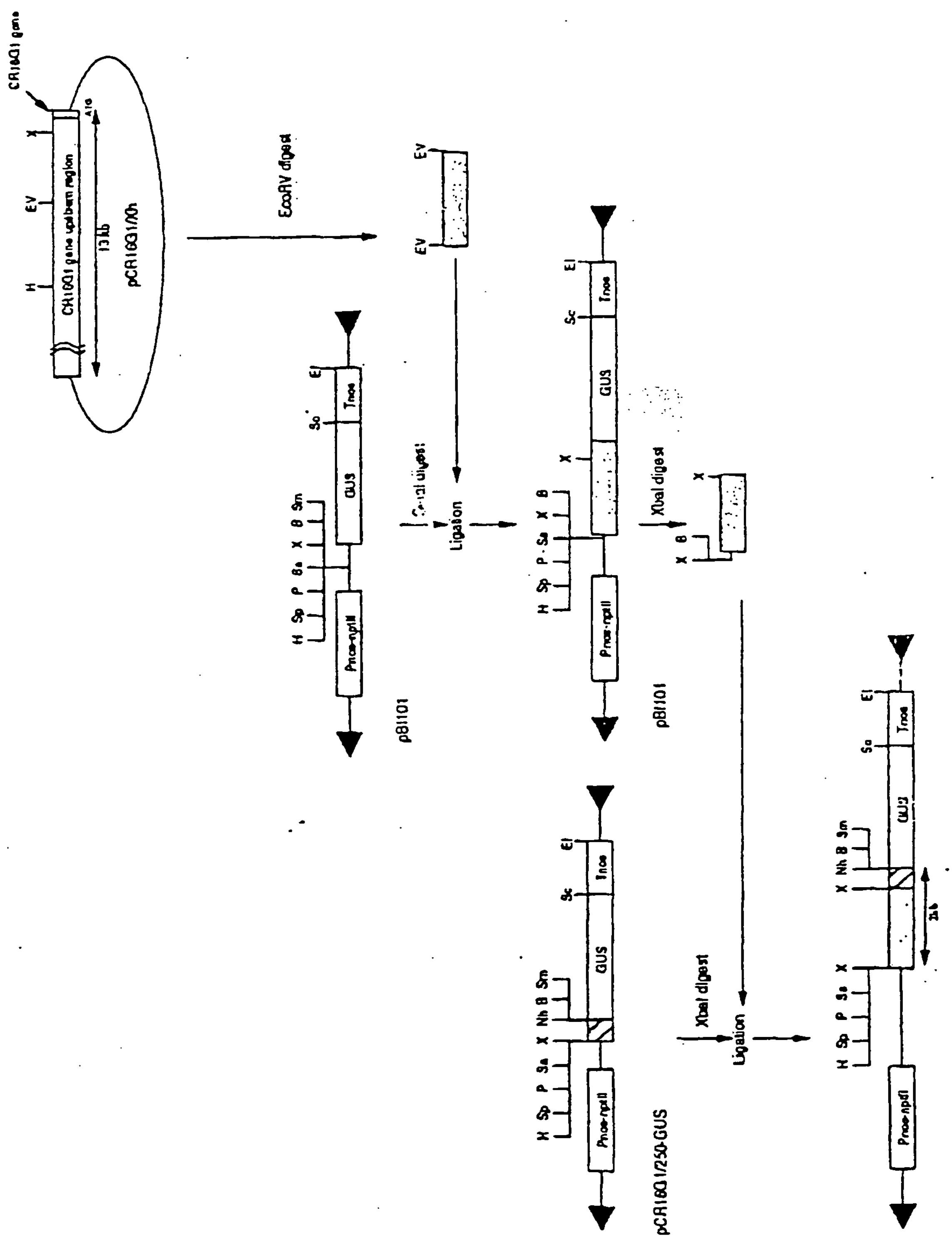

pCR16G1/Xh
EcoRV digest
Ligation
XbaI digest
Ligation
pBI101
pBI101
pCR16G1/250-GUS
pCR16G1/EV-GUS
GUS
Tnos
Pnos-nptII

Fig.6

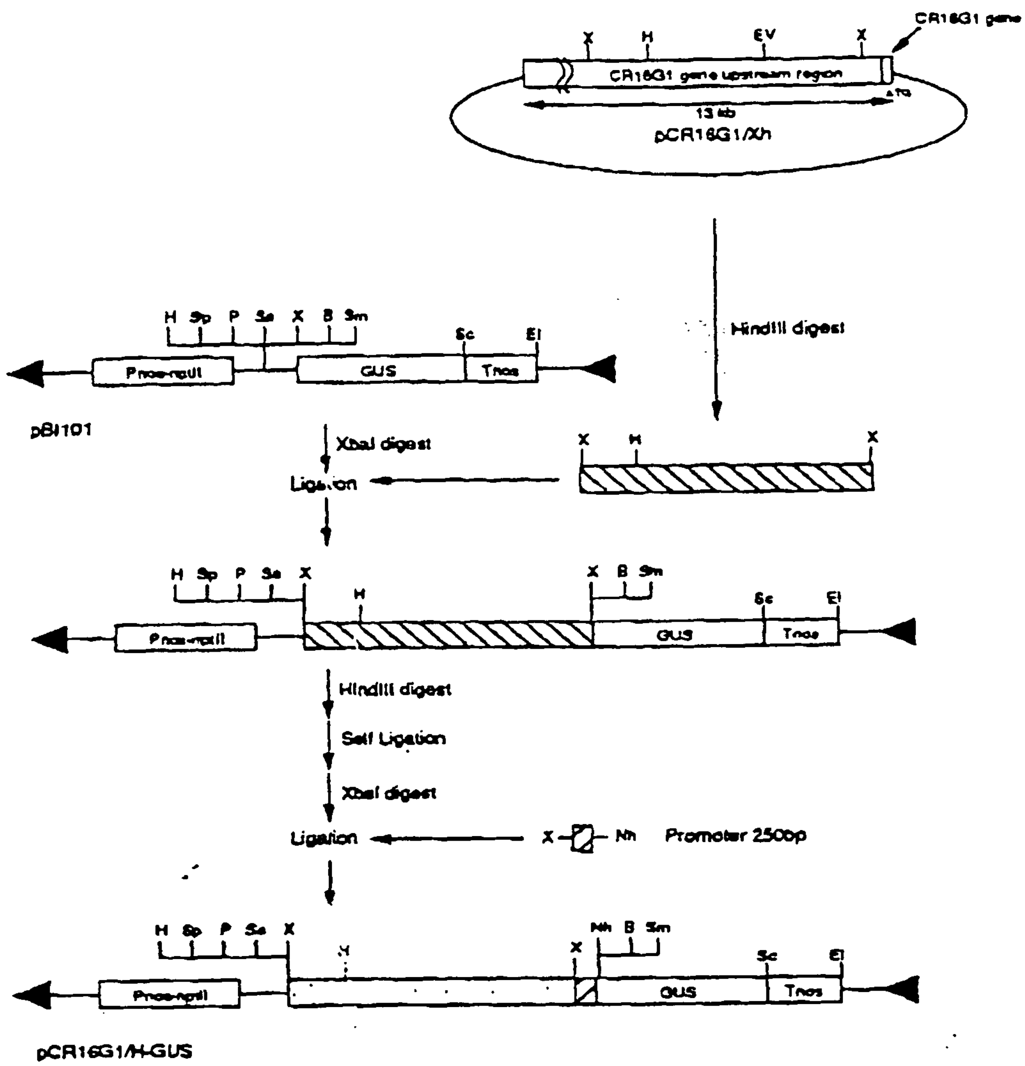
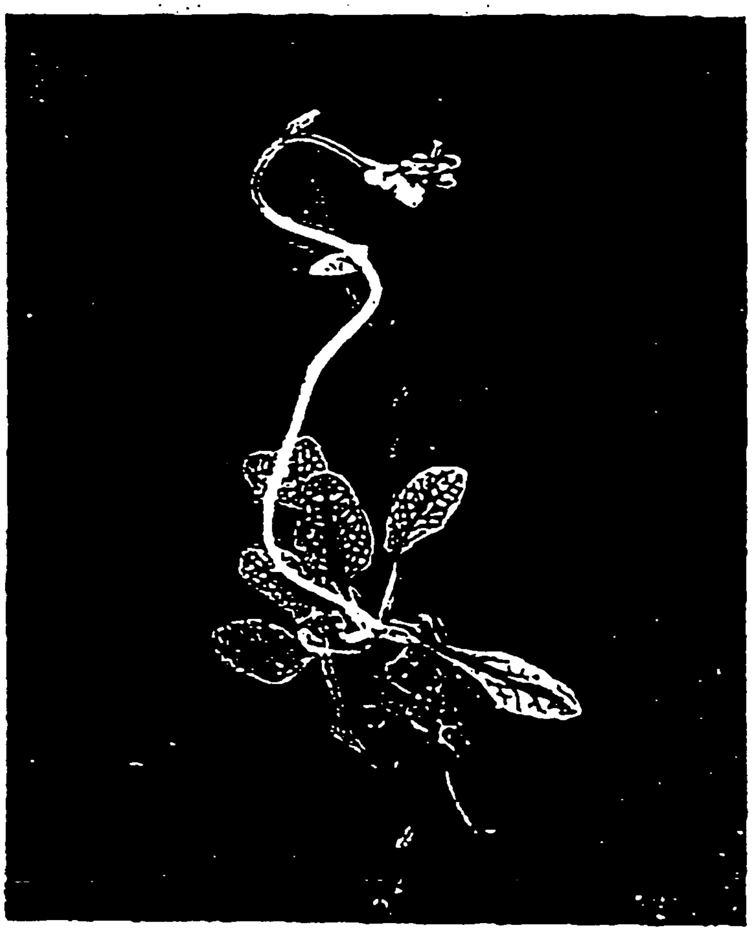

CR16G1 gene
X
H
EV
X
CR16G1 gene upstream region
13 kb
pCR16G1/Xh
HindIII digest
H
Sp
P
Sa
X
B
Sm
Sc
El
Pnos-nptII
GUS
Tnos
pBI101
XbaI digest
Ligation
Ligation
HindIII digest
Self Ligation
XbaI digest
Nh
Promoter 2500p
pCR16G1/H-GUS

Fig.7



■ Non-stained region

□ Stained region

Fig. 9

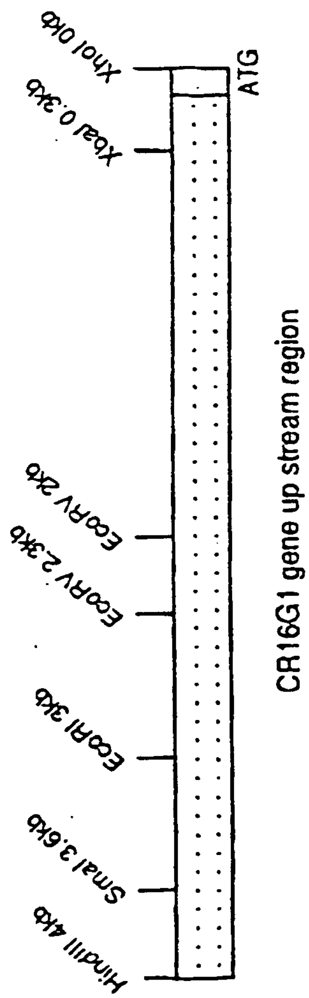

XhoI 0kb
XbaI 0.3kb
EcoRV 2kb
EcoRV 2.3kb
EcoRI 3kb
SmaI 3.6kb
HindIII 4kb
ATG
CR16G1 gene up stream region

Fig.8

■ Non-stained region

□ Stained region

Fig. 10